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Enzymes and Protein
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Determination of Cardiac Troponin I Forms in the Blood of Patients with Acute Myocardial Infarction and Patients Receiving Crystalloid or Cold Blood Cardioplegia

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To determine the forms of cardiac troponin I (cTnI) circulating in the bloodstream of patients with acute myocardial infarction (AMI) and patients receiving a cardioplegia during heart surgery, we developed three immunoenzymatic sandwich assays. The first assay involves the combination of two monoclonal antibodies (mAbs) specific for human cTnI. The second assay involves the combination of a mAb specific for troponin C (TnC) and an anti-cTnI mAb. The third assay was a combination of a mAb specific for human cardiac troponin T (cTnT) and an anti-cTnI mAb. Fifteen serum samples from patients with AMI, 10 serum samples from patients receiving crystalloid cardioplegia during heart surgery, and 10 serum samples from patients receiving cold blood cardioplegia during heart surgery were assayed by the three two-site immunoassays. We confirmed that cTnI circulates not only in free form but also complexed with the other troponin components (TnC and cTnT). We showed that the predominant form in blood is the cTnI-TnC binary complex (IC). Free cTnI, the cTnI-cTnT binary complex, and the cTnT-cTnI-TnC ternary complex were seldom present, and when present, were in small quantities compared with the

binary complex IC. Similar results were obtained in both patient populations studied. These observations are essential for the development of new immunoassays with improved clinical sensitivity and for the selection of an appropriate cTnI primary calibrator.

Cardiac troponin I (cTnI)⁷ is part of the thin filament regulatory complex that causes the ATPase activity of the striated muscle actin-myosin complex to be sensitive to calcium. The troponin complex consists of three subunits: cTnI, cardiac troponin T (cTnT), and troponin C (TnC). These three subunits are called regulatory proteins because each plays a specific role in the regulation of striated and cardiac muscle contraction (1, 2). cTnT binds to tropomyosin and is thought to be responsible for positioning the complex on the thin filament. cTnI is the subunit that inhibits actomyosin ATPase activity, preventing muscle contraction in the absence of Ca^{2+} . TnC is the Ca^{2+} -binding subunit of the troponin complex. The binding of calcium to regulatory sites located in the NH_2 -terminal domain of TnC induces a conformational change that blocks the inhibitory action of cTnI and triggers muscle contraction (3, 4). The interactions between these proteins have been studied extensively. The interaction with the highest affinity ($K_d = 1.3 \times 10^6 \text{ L/mol}$) is the one between cTnI and TnC in the presence of Ca^{2+} (5). The cTnI-cTnT binary complex (IT) is weak, with an associa-

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⁷ Nonstandard abbreviations: cTnI, cardiac troponin I; cTnT, cardiac troponin T; TnC, cardiac or skeletal troponin C; IT, binary cTnI-cTnT complex; IC, binary cTnI-TnC complex; AMI, acute myocardial infarction; mAb, monoclonal antibody; TIC, ternary cTnI-cTnI-TnC complex; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; TC, binary cTnT-TnC complex; A, absorbance; and DL, detection limit.

tion constant of 8.0×10^6 L/mol (6, 7). TnC has four Ca^{2+} -binding sites: two low-affinity sites (sites I and II) located in the NH_2 -terminal domain and two high-affinity sites (sites III and IV) located in the COOH -terminal domain of the molecule (8–10). Sites III and IV perform a structural role. Binding of Ca^{2+} to sites I and II is believed to modify the cTnI-TnC interaction so as to remove the inhibitory action of cTnI (11, 12), leading to the transition of the muscle from the resting to the contracting state. cTnT also interacts with both cTnI and TnC, but the interaction is not as strong as in the cTnI-TnC binary complex (IC) (7).

cTnI has been used as a marker of myocardial injury for almost 10 years, and several assays are available commercially (13–15). At present, cTnI is considered to be the most reliable marker of acute myocardial infarction (AMI), particularly because of its specificity and sensitivity (13–16). cTnI has also been proven to be a specific marker of perioperative myocardial infarction (17, 18), cardiac contusion (19), and cardiac ischemia during cardiac surgery; as such cTnI is used to evaluate and compare different cardioprotective procedures in routine cardiac operations and in heart transplantations (20, 21). Several commercial cTnI assays are currently available; however, the predominant form in which cTnI is released into the bloodstream after myocardial damage has not been determined. Furthermore, cTnI values can differ by a factor 10 or more from one commercial assay to another (22). This can be explained in part by the different monoclonal antibodies (mAbs) used in the assays, which lead to differences in the recognition of the various cTnI forms. Differences in the reference materials used in assay calibration can also explain this divergence. Identification of the circulating forms of cTnI in different cardiac injuries is essential for choosing mAbs adapted to the detection of the prevalent form, which consequently increases the clinical sensitivity of the assay. This knowledge would also be very helpful for establishing a common calibrator, thus enabling harmonization of cTnI values determined with the different commercial assays.

Katrakha et al. (23) used mAbs specific for human cTnI and were able to determine the concentration of the free form and a complexed form. They suggested that the largest part of cTnI is liberated into the bloodstream in the form of a complex, probably with TnC, and that only a small part circulates in the free form. In this study, we developed three two-site immunoassays that allowed us to identify the free form of cTnI as well as each complexed form: the binary complex IC, the binary complex II, and the ternary complex cTnT-cTnI-TnC (TIC). Using these three immunoassays, we analyzed 35 serum samples from patients with AMI and patients receiving a cardioplegia during heart surgery with the objective of identifying the predominant cTnI form(s) released into the bloodstream after myocardial damage.

Materials and Methods

SAMPLES

Blood samples from 15 Q-wave AMI patients were collected at different times (between 0 and 48 h) after their arrival at the hospital for chest pain. For all samples, arrival at the hospital was between 2 and 6 h after the onset of chest pain. The samples were centrifuged after collection, and the serum was stored immediately at -20°C until use. Diagnosis of AMI was confirmed by coronary angiography, and patients were treated with primary percutaneous transluminal coronary angioplasty.

Blood samples from 20 patients receiving either crystalloid cardioplegia (10 patients) or cold blood cardioplegia followed by warm reperfusion (10 patients) were collected just before cardiopulmonary bypass and after aortic unclamping at 6, 9, 12, and 24 h and daily thereafter for 5 days. Samples were centrifuged after collection, and serum was stored immediately at -20°C until use (24). Patients receiving cardioplegia had first undergone elective coronary artery bypass grafting. Not included in this group were patients requiring only one distal anastomosis, patients with an ejection fraction <0.30 , patients undergoing a repeat operation, and patients with concomitant heart valve disease or unstable angina.

Blood samples from 12 healthy donors with no history of heart disease were centrifuged after collection, and the serum was stored immediately at -20°C until use. These samples were confirmed cTnI negative by the ACCESS[®] immunoassay system (Sanofi Diagnostics Pasteur-Beckman Instruments) and cTnT negative by the ELECSYS[®] immunoassay system (Boehringer Mannheim).

Because leftover blood from routine collections was used for all samples and no patient identifiers were used, informed consent was deemed unnecessary.

mAbs

Different mAb combinations were tested in a sandwich enzyme immunoassay in an attempt to find the optimal mAb pairs for the measurement of the various cTnI forms. Four antibodies were selected to perform the immunoassays: mAb 1, monoclonal mouse anti-cTnI (Sanofi Recherche) (25); mAb 2, monoclonal mouse anti-cTnI (Sanofi Recherche) (25); mAb 2I-11, monoclonal mouse anti-TnC (Spectral Diagnostics); mAb 7G-7, monoclonal mouse anti-cTnT (Hytest), which recognizes fragment 60–71 on the cTnT molecule. mAbs 1 and 7G-7 were used as detection antibodies labeled with horseradish peroxidase (HRP). mAbs 2 and 2I-11 were used as capture antibodies immobilized onto 96-well microtitration plates.

Labeling of antibodies with HRP. The HRP used to label detection antibodies was obtained from Boehringer Mannheim Biochemicals. We labeled mAbs 1 and 7G-7 by mixing 3 mg of each mAb with 2.25 mg of oxidized HRP and incubating the mixture for 2 h in the dark under gentle shaking at room temperature. Labeled mAbs were stabilized with 4 g/L sodium borohydride (Aldrich

Chemical) and incubated for 2 h at 4 °C in the dark under gentle shaking. After dialysis overnight at 4 °C in phosphate-buffered saline (PBS Dulbecco; Seromed), the conjugates were diluted in 550 mL/L glycerol and stored at -20 °C.

Coating of the microtiter plates. The capture mAbs 2 and 2I-11 were immobilized onto 96-well microtitration plates (Nunc Maxisorp) by physical adsorption. Wells were coated with 250 μ L of the mAb diluted to 5 mg/L in PBS Dulbecco. After incubation overnight at 4 °C, the microplates were washed three times with 300 μ L of PBS Dulbecco containing 1 mL/L Tween 20 (Sigma Chemical). They were then saturated with 250 μ L of Tris-buffered saline (TBS) containing 10 g/L bovine serum albumin (BSA; Sigma), incubated for 1 h at 37 °C, and washed three times with 300 μ L of PBS Dulbecco containing 1 mL/L Tween 20.

IMMUNOASSAYS

Assay description. Four two-site immunoassays were performed in coated 96-well microtitration plates. The first assay, the total cTnI assay for detection of free and complexed cTnI, was performed with two anti-cTnI mAbs: mAb 2 coated onto the microplates was used as the capture mAb, and the mAb 1-HRP conjugate diluted 1/5000 in PBS Dulbecco containing 10 g/L BSA and 1 mL/L Tween 20 was used as the detection mAb. The second assay, the IC-TIC assay, was performed with an anti-cTnI mAb associated with an anti-TnC mAb. mAb 2I-11 coated onto the microplates was used as the capture antibody, and the mAb 1-HRP conjugate diluted 1/5000 in TBS containing 10 g/L BSA and 1 mL/L Tween 20 was used as the detection antibody. The third assay, the IT-TIC assay, used an anti-cTnI mAb and an anti-cTnT mAb. mAb 2 coated onto the microplates was used as the capture antibody, and the mAb 7G-7-HRP conjugate diluted 1/200 in TBS containing 10 g/L BSA and 1 mL/L Tween 20 was used as the detection antibody. The fourth assay, the cTnT-TnC binary complex (TC)-TIC assay, used an anti-TnC mAb and an anti-cTnT mAb. mAb 2I-11 coated onto the microplates was used as the capture antibody, and the mAb 7G-7-HRP conjugate diluted 1/200 in TBS containing 10 g/L BSA and 1 mL/L Tween 20 was used as the detection antibody.

Assay protocol. One hundred microliters of serum sample or calibrator and 100 μ L of the appropriate mAb-HRP conjugate were added to each coated well and incubated for 6 h at 4 °C under gentle shaking for the total cTnI assay, the IT-TIC assay, and the TC-TIC assay, and for 1 h 30 min at 4 °C for the IC-TIC assay. After the plates were washed five times with PBS Dulbecco containing 1 mL/L Tween 20, 200 μ L of the substrate o-phenylenediamine/hydrogen peroxide (Sanofi Diagnostics Pasteur)

was added to each well and incubated for 15 min in the dark at room temperature. The reaction was stopped by the addition of 50 μ L 2 mol/L H₂SO₄. The absorbance was measured at 492 nm with the LP400 microplate reader (Sanofi Diagnostics Pasteur). Samples with an absorbance >3.0, the limit of the microplate reader, were diluted in normal human serum (Scantibodies Laboratory), which was confirmed cTnI negative by the ACCESS immunoassay system and cTnT negative by the ELECSYS immunoassay system.

CALIBRATOR SOLUTION PREPARATIONS

Calibrator solutions were prepared for each cTnI form: free cTnI, the TIC ternary complex, the IC binary complex, and the IT binary complex.

TIC complex calibrator solutions. We used troponin complex TIC from human heart tissue (Hytest). The concentration of the TIC complex stock solution was 0.31 g/L in cTnI, as determined by gel scanning by the manufacturer. We prepared the TIC complex calibrator solutions by diluting the TIC complex stock solution in the Scantibodies normal human serum described above to give 0, 0.1, 0.5, 1, 2, 5, 10, 30, and 50 μ g cTnI/L.

Free cTnI calibrator solutions. Free cTnI calibrator solutions were prepared with cTnI (purity >95%) from human heart tissue (Hytest). The concentration of the cTnI stock solution, determined spectrophotometrically, was 0.05 g/L. The cTnI stock solution was diluted with the Scantibodies normal human serum described above to give 0, 0.1, 0.5, 1, 2, 5, 10, 30, and 50 μ g/L.

IC complex formation and calibrator solution preparation. TnC (purity >98%) and cTnI from human heart tissue were used to form the IC binary complex. A sixfold molar excess of TnC was added to cTnI diluted in the Scantibodies normal human serum containing 2 mmol/L CaCl₂ (23). The mixture was incubated for 30 min at 4 °C under gentle shaking. Using the stock solution of IC complex at 500 μ g/L in cTnI, we made the following dilutions in the Scantibodies normal human serum containing 2 mmol/L CaCl₂: 0, 0.1, 0.5, 1, 2, 5, 10, 30, and 50 μ g cTnI/L.

IT complex formation and calibrator solution preparation. cTnT (purity >98%) from human heart tissue (Scripps) and cTnI purified from human heart tissue (Hytest) were used to form the binary complex IT. A sixfold molar excess of cTnT was added to cTnI diluted in the Scantibodies normal human serum containing 2 mmol/L CaCl₂. This mixture was incubated for 30 min at 4 °C under gentle shaking. Using the stock solution of IT complex at 500 μ g/L in cTnI, we made the following dilutions in the Scantibodies normal human serum containing 2 mmol/L CaCl₂: 0, 0.1, 0.5, 1, 2, 5, 10, 30, and 50 μ g cTnI/L.

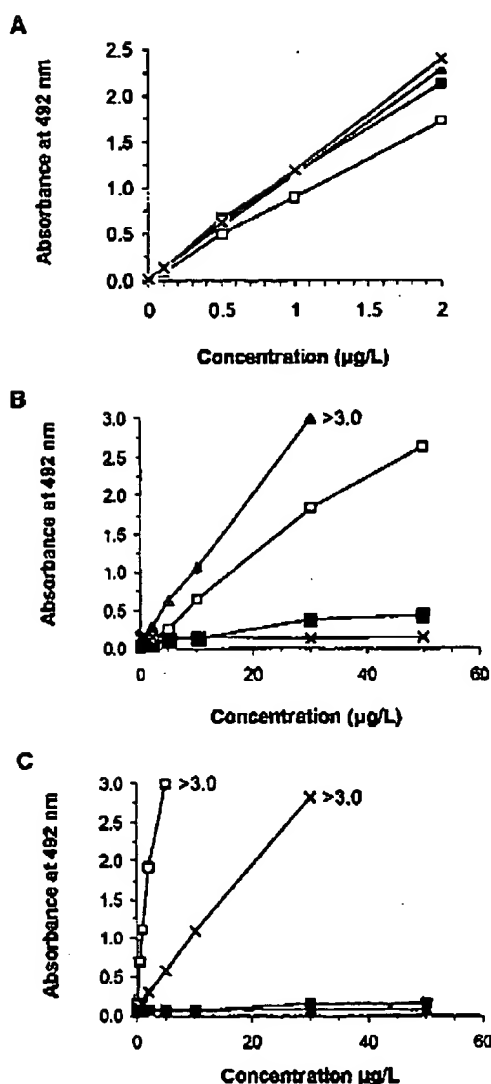


Fig. 1. Calibration curves for the three immunoassays with each cTnI form. (A), Total cTnI assay. \square , calibration with the TIC complex diluted in normal human serum (linear regression: $y = 0.853x + 0.027$, $r = 0.99$); \blacksquare , calibration with free cTnI diluted in normal human serum (linear regression: $y = 1.055x + 0.062$, $r = 0.99$); \blacktriangle , calibration with the IC complex diluted in normal human serum containing a sixfold molar excess of TnC (linear regression: $y = 1.130x + 0.021$, $r = 0.99$); \times , calibration with the IT complex diluted in normal human serum containing a sixfold molar excess of cTnT (linear regression: $y = 1.186x + 0.016$, $r = 0.99$). (B), IC-TnI assay. \square , calibration with the TIC complex diluted in normal human serum (linear regression: $y = 0.054x + 0.028$, $r = 0.89$); \blacksquare , calibration with free cTnI diluted in normal human serum (linear regression: $y = 0.008x + 0.039$, $r = 0.94$); \blacktriangle , calibration with the IC complex diluted in normal human serum containing a sixfold molar excess of TnC (linear regression: $y = 0.099x + 0.037$, $r = 0.99$); \times , calibration with the IT complex diluted in normal human serum containing a sixfold molar excess of cTnT (linear regression: $y = 0.0003x + 0.13$, $r = 0.032$). (C), IT-TnI assay. \square , calibration with the TIC complex diluted in normal human serum

SENSITIVITY OF THE IMMUNOASSAYS

To illustrate the sensitivity of our immunoassays for each calibrator, we calculated the response curve slope for each calibration curve (26, 27).

Results

CALIBRATION OF THE THREE TWO-SITE IMMUNOASSAYS FOR cTnI FORMS

To choose a common calibrator for our assays, we first tested various calibrators diluted in normal human serum: free cTnI, the IC binary complex prepared with a sixfold molar excess of TnC, the IT binary complex prepared with a sixfold molar excess of cTnT, and the TIC ternary complex.

The calibration curves for the total cTnI assay is shown in Fig. 1A. With this assay, we detected all forms of cTnI: free cTnI, the IC binary complex, the IT binary complex, and the TIC ternary complex. Free cTnI and the binary complexes IC and IT were detected with the same sensitivity [free cTnI, slope ($A \cdot L/\mu g$) = 1.055; complex IC, slope ($A \cdot L/\mu g$) = 1.130; complex IT, slope ($A \cdot L/\mu g$) = 1.186, where A is the absorbance at 492 nm]. The sensitivity for the complex TIC was slightly lower: slope ($A \cdot L/\mu g$) = 0.853.

The calibration curves for the IC-TnI assay are shown in Fig. 1B. With this assay, we detected two cTnI forms: the IC binary complex, and the TIC ternary complex. The slope ($A \cdot L/\mu g$) obtained for the complex IC was 0.099. The sensitivity for the complex TIC was lower: slope ($A \cdot L/\mu g$) = 0.054. Free cTnI diluted in normal human serum was detected only faintly at high concentrations. A test with free cTnI diluted either in serum or in succinate buffer at 100 and 1000 $\mu g/L$ showed that the signal was increased only in serum, which suggested that the normal human serum we used probably contained TnC from skeletal muscle, which formed an IC complex when cTnI was present at rather high concentrations ($\geq 30 \mu g/L$). When the IT complex was tested with this assay, the signal was the same as that for the nonspecific binding.

The calibration curves for the IT-TnI assay are shown in Fig. 1C. This assay also detected two forms of cTnI: the IT binary complex and the TIC ternary complex. The sensitivity ($A \cdot L/\mu g$) of this assay for the TIC complex was 0.579, whereas the sensitivity of the assay for the IT complex was lower: slope ($A \cdot L/\mu g$) = 0.092. For free cTnI and IC complex, the signal detected was the same as that for the nonspecific binding.

(linear regression: $y = 0.579x + 0.319$, $r = 0.93$); \blacksquare , calibration with free cTnI diluted in normal human serum (linear regression: $y = 0.002x + 0.054$, $r = 0.96$); \times , calibration with the IT complex diluted in normal human serum containing a sixfold molar excess of cTnT (linear regression: $y = 0.092x + 0.083$, $r = 0.99$); \blacktriangle , calibration with the IC complex diluted in normal human serum containing a sixfold molar excess of TnC (linear regression: $y = 0.0003x + 0.06$, $r = 0.33$).

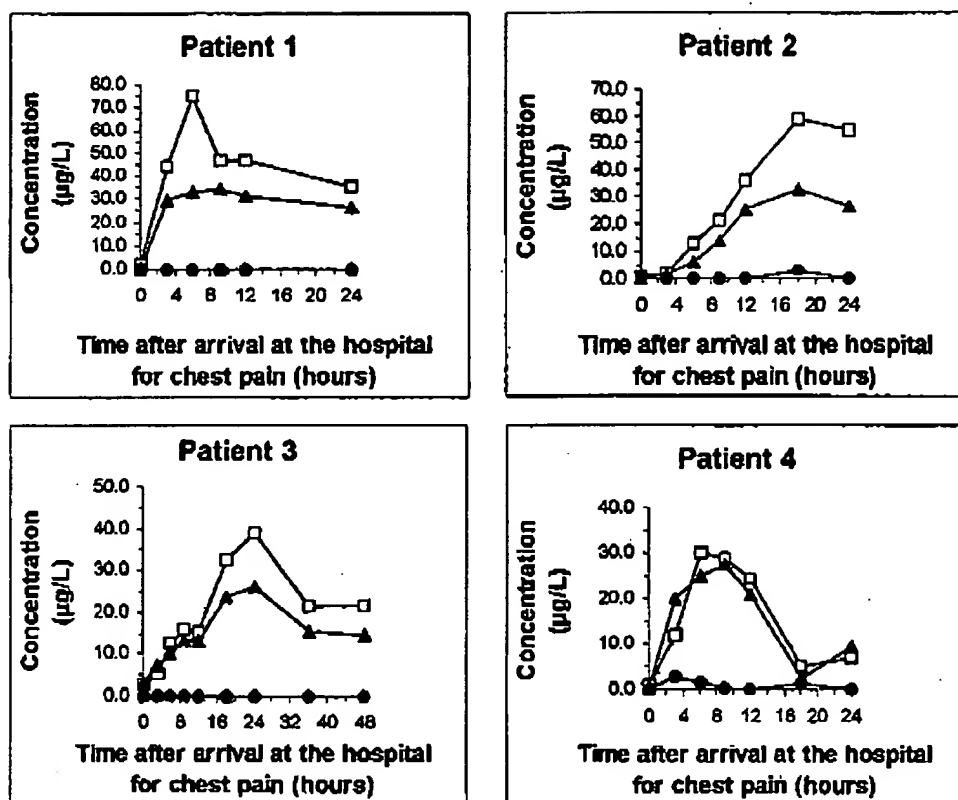


Fig. 2. Kinetics of the release of the cTnI forms in the sera of AMI patients 1-4.

Concentrations of cTnI forms were calculated from the TIC calibration curve. ▲, total cTnI assay; □, IC-TIC assay; ●, IT-TIC assay.

The total cTnI assay was more sensitive than the IC-TIC assay for detection of the IC and the TIC complexes and more sensitive than the IT-TIC assay for the detection of the IT complex. The total cTnI assay and the IT-TIC assay detected the TIC complex with approximately the same sensitivity.

We decided to standardize our three assays with the only common form of cTnI detected, namely the TIC complex diluted in normal human serum. The detection limit (DL) was determined by testing 10 S_0 samples (normal human serum without the TIC complex) and 10 S_1 samples (complex TIC diluted in normal human serum to a concentration of 0.1 μg cTnI/L) in the three assays [$\text{DL} = [(2 \times \text{SD } S_0) / (\text{mean absorbance } S_1 - \text{mean absorbance } S_0)] \times [S_1]$]. The DLs for the total cTnI assay, the IC-TIC assay, and the IT-TIC assay were 0.01, 0.70, and 0.03 $\mu\text{g/L}$, respectively. The mean concentration of the cTnI forms in the 12 normal human sera was found to be below the DL for each assay.

With the TC-TIC assay, we were able to detect the TIC ternary complex analytically, but with poor sensitivity. When we reconstituted the TC binary complex in buffer or in serum, we were unable to detect it.

ASSAY OF cTnI FORMS IN SERA FROM 15 AMI PATIENTS
Serum samples from 15 AMI patients collected at different times after their arrival at the hospital for chest pain were assayed simultaneously with the three assays calibrated with the TIC complex. No matrix effects were introduced into the assay by dilution of the samples. Fig. 2 shows the kinetics of the release of the cTnI forms in the sera of AMI patients 1-4. These results are typical of those obtained for the 15 AMI patients. The majority of samples were positive in the total cTnI assay and the IC-TIC assay; only a few samples were positive in the IT-TIC assay. Of the 15 patients, one had higher values in the IT-TIC assay than in the IC-TIC assay. Fig. 3 illustrates the mean results for the 15 AMI patients, determined by the three assays at each sampling time after their arrival at the hospital. In the total cTnI assay, all patients were positive between 3 and 18 h after their arrival at the hospital, and 11 of 15 patients were positive between their arrival at the hospital and 3 h later. We observed a peak at 12 h after their arrival at the hospital, i.e., between 14 and 18 h after the onset of chest pain, the corresponding concentration was 38.26 ± 26.02 $\mu\text{g/L}$ (mean \pm SD). The results followed a similar pattern with the IC-TIC assay. The results for all patients were

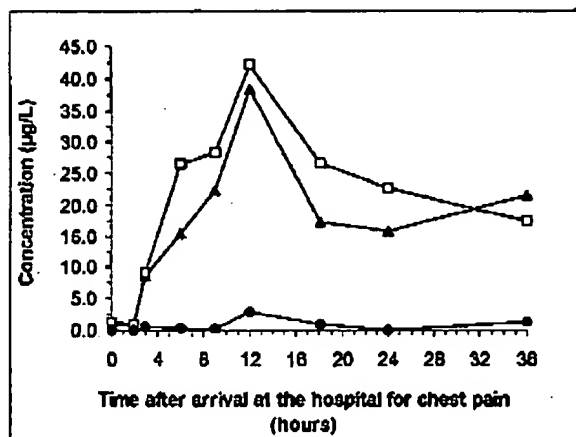


Fig. 3. Kinetics of release of the cTnI forms in the sera of the 15 AMI patients.

Concentrations of the cTnI forms were calculated from the TIC calibration curve. Each sample was tested in the three assays. Each point represents the average of results from 15 AMI patient samples. ▲, total cTnI assay; □, IC-TIC assay; ●, IT-TIC assay.

positive between 6 and 18 h after their arrival at the hospital, and the results for 6 of the 15 patients were positive for samples taken between their arrival at the hospital and 3 h later. A peak was observed 12 h after their arrival at the hospital; the corresponding concentration was $42.15 \pm 31.73 \mu\text{g/L}$. The results obtained with the IT-TIC assay showed that it was possible to detect the IT complex and/or the TIC complex in serum samples

from AMI patients; however, the concentrations were very low compared with those found by the total cTnI assay and the IC-TIC assay. Only 2 of 15 patients were positive between their arrival at the hospital and 3 h later. There was a peak at 12 h after the patients' arrival at the hospital, but the corresponding concentration was low: $2.8 \pm 18.43 \mu\text{g/L}$.

ASSAY OF cTnI FORMS IN SERA FROM 20 PATIENTS RECEIVING CARDIOPLEGIA

We also tested serum samples collected from 10 patients receiving crystalloid cardioplegia and from 10 patients receiving cold blood cardioplegia. Serum samples were collected just before cardiopulmonary bypass and after aortic unclamping at 6, 9, 12, and 24 h and daily thereafter for 5 days. Serum samples were tested simultaneously by the three sandwich immunoassays calibrated with the TIC complex. No matrix effects were introduced into the assay by dilution of the samples. Fig. 4 shows the kinetics of the appearance of the cTnI forms in the sera of six patients. Patients 5, 6, and 7 received crystalloid cardioplegia, whereas patients 8, 9, and 10 received cold blood cardioplegia. Fig. 5 illustrates the mean values at each sampling time of the cTnI forms in serum samples from the patients receiving crystalloid cardioplegia (Fig. 5A) or cold blood cardioplegia (Fig. 5B). As expected, the concentrations were lower than those in the sera from AMI patients. With the total cTnI assay and the IC-TIC assay, maximum

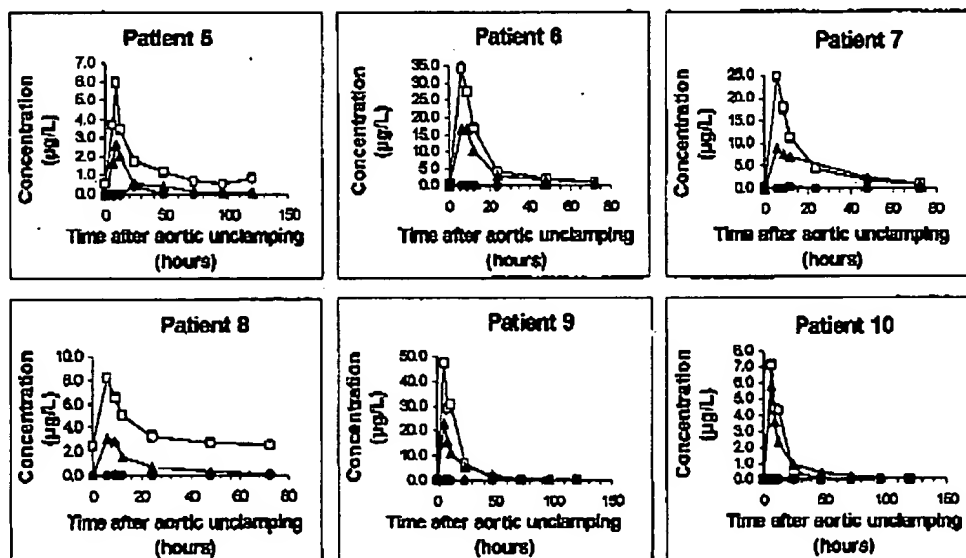


Fig. 4. Kinetics of the release of the cTnI forms in the sera of patients 5, 6, and 7 (representative results for patients receiving crystalloid cardioplegia during heart surgery) and patients 8, 9, and 10 (representative results for patients receiving cold blood cardioplegia during heart surgery).

Concentrations of the cTnI forms were calculated from the TIC calibration curve. ▲, total cTnI assay; □, IC-TIC assay; ●, IT-TIC assay.

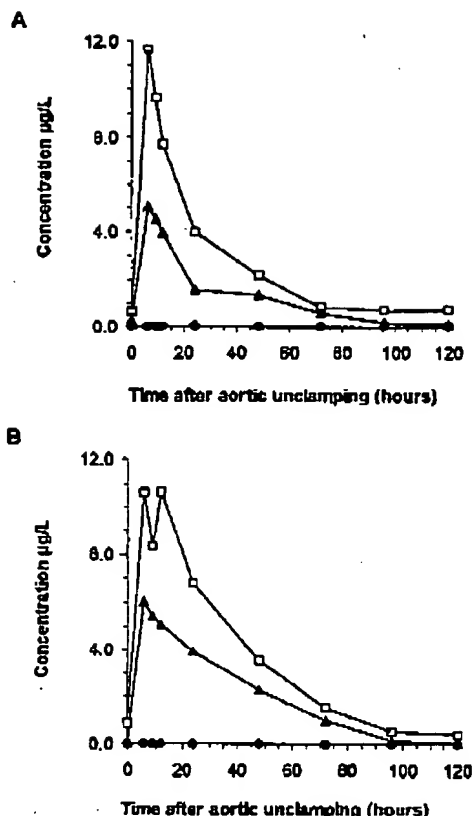


Fig. 5. Kinetics of the release of the cTnI forms in the sera of heart surgery patients.

Concentrations of the cTnI forms were calculated from the TIC calibration curve. Δ , total cTnI assay; \square , IC-TIC assay; \bullet , IT-TIC assay. (A), each point represents the average of results from 10 patients receiving crystalloid cardioplegia during heart surgery. (B), each point represents the average of results from 10 patients receiving cold blood cardioplegia during heart surgery.

concentrations were observed for both populations at 6 h after aortic unclamping. For patients who received crystalloid cardioplegia, the concentrations observed at the peak were $5.09 \pm 4.36 \mu\text{g/L}$ in the total cTnI assay and $11.66 \pm 10.38 \mu\text{g/L}$ in the IC-TIC assay. For patients who received cold blood cardioplegia, the concentrations observed at the peak were $6.02 \pm 7.18 \mu\text{g/L}$ in the total cTnI assay and $10.66 \pm 14.11 \mu\text{g/L}$ in the IC-TIC assay. The binary IT complex and the ternary TIC complex were not present in these samples, as illustrated by the results with the IT-TIC assay. In Fig. 5B, there were two peaks at 6 and at 12 h after aortic unclamping for the IC and/or TIC complexes measured using the IC-TIC assay for the patients who had received cold blood cardioplegia. This can probably be explained by the fact that the concentrations of the cTnI forms in the serum of one patient (Fig. 4, patient 9) were so high that the profiles of the curves were modified.

RESULTS OBTAINED FOR AMI PATIENTS AND PATIENTS RECEIVING A CARDIOPLEGIA WITH THE TOTAL cTnI ASSAY AND THE IC-TIC ASSAY CALIBRATED WITH THE IC COMPLEX

After analyzing the results obtained with our three immunoassays calibrated with the TIC ternary complex, we decided to recalculate the concentrations of the test samples measured by both the total cTnI assay and the IC-TIC assay calibrated with the IC binary complex (Fig. 1, A and B).

The results obtained with AMI patients and patients receiving either cold blood or crystalloid cardioplegia are shown in Fig. 6. The profiles of the troponin release observed with the total cTnI assay and the IC-TIC assay in AMI patients (Fig. 6A) are perfectly superposed, with a peak at 12 h after their arrival at the hospital. The corresponding concentrations were $26.68 \pm 18.76 \mu\text{g/L}$ in the total cTnI assay and $26.57 \pm 23.18 \mu\text{g/L}$ in the IC-TIC assay. The same results were obtained in patients receiving cold blood cardioplegia (Fig. 6B). The profiles of the troponin release obtained with the total cTnI assay and the IC-TIC assay are superposed, with a peak at 6 h after aortic unclamping and corresponding concentrations of $4.62 \pm 5.73 \mu\text{g/L}$ in the total cTnI assay and $4.90 \pm 7.23 \mu\text{g/L}$ in the IC-TIC assay. The profiles of the troponin release observed with the total cTnI assay and the IC-TIC assay in patients receiving crystalloid cardioplegia are not superposed: between 6 and 24 h after aortic unclamping, concentrations measured by the IC-TIC assay were higher than those measured by the total cTnI assay. The maximum concentrations measured were $2.89 \pm 1.022 \mu\text{g/L}$ by the total cTnI assay and $3.94 \pm 2.92 \mu\text{g/L}$ by the IC-TIC assay (Fig. 6C).

Discussion

The commercially available cTnI assays detect myocardial injury with very high specificity and sensitivity (13–15). Because cTnI interacts with other contractile proteins of the thin filament and in particular with the two other troponins, cTnT and TnC, it was of great interest to determine whether cTnI circulates in the free form or as a complex shortly after its release. We developed three immunoassays to detect the different cTnI forms: free cTnI and the IC, IT, and TIC complexes. We also developed a fourth assay to measure the TC and/or TIC forms, using one mAb directed against cTnT and another directed against TnC. The TIC complex was detected weakly; the TC complex, however, was not detected even when present at high concentrations. Ingraham and Swenson (7) reported an association constant for TnC and TnT from rabbit fast skeletal muscle of $4 \times 10^7 \text{ L/mol}$. Nevertheless, they indicated that TnT has a tendency to form aggregates in solution, and it may be in equilibrium with dimers, trimers, and tetramers. The presence of TnT aggregates could have prevented TnT from associating with TnC to form the TC complex, and this probably explains why we did not detect the TC complex.

To calibrate the three immunoassays, we tested all the

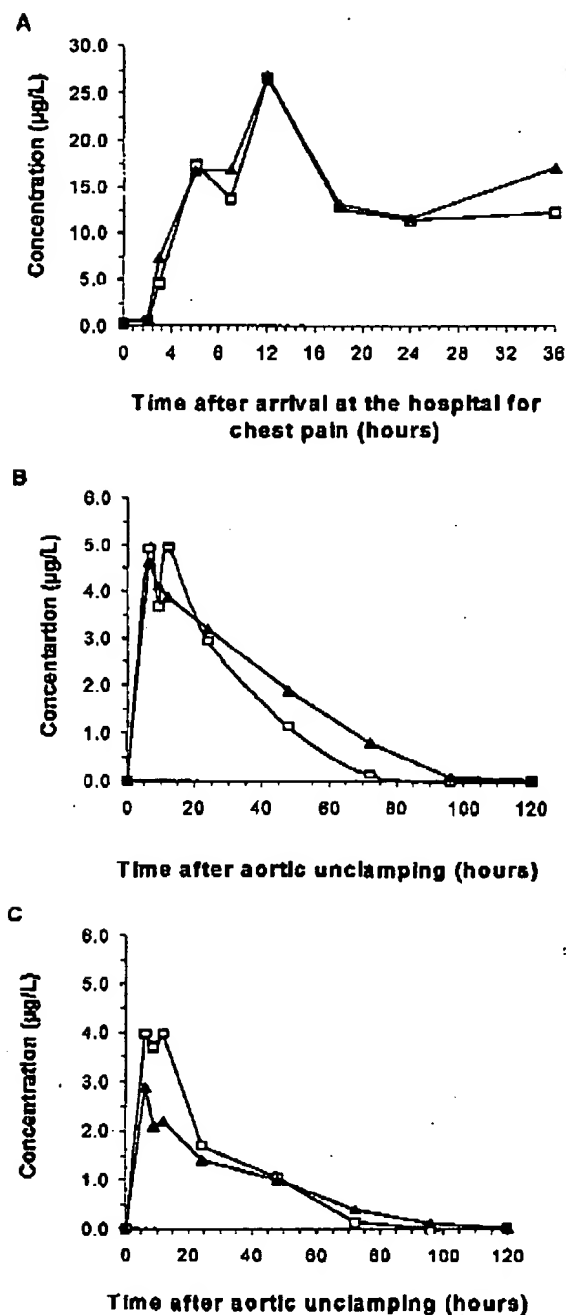


Fig. 6. Kinetics of release of the cTnI forms in the sera of patients with cardiac damage.

Concentrations of the cTnI forms were calculated from the IC calibration curve. A, total cTnI assay; □, IC-TnI assay. (A), each point represents the average of results from 15 AMI patient samples. (B), each point represents the average of results from 10 patients receiving cold blood cardioplegia during heart surgery. (C), each point represents the average of results from 10 patients receiving crystalloid cardioplegia during heart surgery.

troponin forms: I, IC, IT, and TIC. Because the TIC complex form was the only common calibrator, we decided to use it to calibrate the assays.

Using the three immunoassays developed in this study, we measured the kinetics of the release of free cTnI, IC, IT, and TIC in serum samples from AMI patients at different times after their arrival at the hospital. Among the 15 AMI patients, all sera were found to be positive in the total cTnI assays and in the IC-TIC assay, whereas the sera from only 2 of the 15 patients were weakly positive when measured with the IT-TIC assay. Because the IT-TIC assay detects only the IT and/or TIC forms, we conclude that the IT and/or TIC forms exist only in a low proportion of AMI patients, and at a very low concentration. The presence of the IT and/or TIC forms in the serum samples of two AMI patients might be indicative of a poor prognostic; this must be confirmed in a larger population. The patterns of troponin release measured by the total cTnI assay and the IC-TIC assay were the same in patients receiving cardioplegia during heart surgery as those for AMI patients. Neither IT nor TIC was detected in the serum of the 20 patients with heart surgery by the IT-TIC assay. As suggested by the work of Ettevent et al. (20), the increases in the concentrations of the troponin forms measured by the total cTnI assay and the IC-TIC assay were probably caused by cardiac ischemia during heart surgery.

Because TIC was not detected in the serum samples from most of the patients, even when the most sensitive assay was used, we concluded that the IT and TIC complexes were not predominant forms in either patient population studied. Therefore, the cTnI forms measured in the patients' sera by the total cTnI assay were probably free cTnI and/or the IC complex, and the IC-TIC assay probably only detected the IC complex. Using the TIC complex to calibrate the assays, we found a slightly higher mean concentration of cTnI forms in the serum samples when the IC-TIC assay was used than when the total cTnI assay was used. Moreover, the release kinetics of the cTnI forms observed for both patient populations studied showed the same profile when measured with the total cTnI assay as with the IC-TIC assay. In other words, the release patterns observed for free cTnI and/or the IC complex in the total cTnI assay and for IC complex in the IC-TIC assay were the same. On the basis of these findings, we hypothesized that the total cTnI assay and the IC-TIC assay were detecting the same molecule, namely the IC complex. Indeed, when we calibrated both the total cTnI assay and the IC-TIC assay with the IC complex, we found that the release profiles for the cTnI forms measured by the two assays in serum samples from AMI patients and from patients receiving cold blood cardioplegia were superposed. We therefore strongly suggest that the IC complex is the main circulating form for these pathologies. For patients receiving crystalloid cardioplegia, the release profiles were not well superposed. This discrepancy could be attributed to a component of

the crystalloid cardioplegia solution, which might interfere with our immunoassays. This remains to be confirmed.

Taken together, our results suggest that troponin forms are not released in the order expected on the basis of their molecular weights, i.e., cTnI first, because of its low molecular weight, then IC, and then TIC. We would also have expected to find TIC released first, then IC, and finally cTnI, indicative of complex dissociation after the release into the bloodstream. In fact, an equilibrium probably exists between all the possible released forms, and the form that predominates, the IC complex, is the one with the highest association constant and, therefore, the most stable.

Katrakha et al. (23), using one pair of anti-cTnI mAbs that recognize only free cTnI and another pair of anti-cTnI mAbs that recognize free and complexed cTnI in the presence of EDTA, showed that the main part of cTnI is released as a complex, without specifying whether the isoform released is IC or TIC. The use of three immunoassays able to detect all of the cTnI complexes with mAbs directed against cTnI, cTnI, and TnC and the absence of detection of the IT and/or TIC forms in the sera of most patients allowed us to conclude that IC is the predominant complex circulating, and not TIC. These results corroborate the findings that cTnI is stabilized by TnC (28).

The findings reported here are of a great importance for the standardization of the commercially available cTnI assays. Because IC is the main circulating form and one of the most stable, it is likely to be the best form to use as a standard for the different assays. That would be the first step along the way to standardization. Because different mAbs are used in the different commercially available assays and because our results as well as those of Katrakha et al. (23) suggest that low amounts of other cTnI forms (free cTnI, TIC, and/or IT) are released, it seems important to use a representative panel of serum samples from patients in addition to a primary standard. This panel could be used by each manufacturer to determine the clinical sensitivity of the assay.

In conclusion, we have shown that cTnI circulates in blood mainly as the IC complex form in AMI and heart surgery patients shortly after the release of troponin. Free cTnI, IT, and/or TIC forms were found in the blood of a few AMI patients, but at low concentrations compared with IC. Therefore, a cTnI assay must have the following two features: it must be able to recognize the predominant form IC; and it must be able to recognize the cTnI forms released into the bloodstream under different clinical conditions, i.e., in patients with different types of myocardial injury. In this investigation, we analyzed the release of cTnI forms in only two patient populations: AMI patients and patients receiving cardioplegia during heart surgery. We are now planning a study on patients with unstable angina to see whether they show the same release pattern as AMI patients or, which is more likely,

the same pattern as patients who have undergone heart surgery, our aim being to investigate whether we can associate the different cTnI forms with specific pathologies.

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References

1. Zot AS, Potter TD. Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. *Annu Rev Biophys Chem* 1987;16:535-59.
2. Leavis P, Gergely J. Thin filament proteins and thin filament-linked regulation of vertebrate muscle contraction. *CRC Crit Rev Biochem* 1984;16:235-305.
3. Farah CS, Miyamoto CA, Ramos CHI, da Silva ACR, Quaggio RB, Fujimori K, et al. Structural and regulatory functions of the NH₂- and COOH-terminal regions of skeletal muscle troponin I. *J Biol Chem* 1994;269:5230-40.
4. Farah CS, Reinach F. The troponin complex and regulation of muscle contraction. *FASEB J* 1995;9:755-67.
5. Liao R, Wang CK, Cheung HC. Coupling of calcium to the interaction of troponin I with troponin C from cardiac muscle. *Biochemistry* 1994;33:12729-34.
6. Malnic B, Farah CS, Reinach FC. Regulatory properties of the NH₂- and COOH-terminal domains of troponin I. *J Biol Chem* 1998;273:10594-601.
7. Ingraham RH, Swenson CA. Binary interactions of troponin subunits. *J Biol Chem* 1984;259:9544-8.
8. Morimoto S, Ohtsuki I. Ca²⁺ binding to cardiac troponin C in myofilament lattice and its relation to the myofibrillar ATPase activity. *Eur J Biochem* 1994;226:597-602.
9. Sorenson MM, da Silva ACR, Gouveia CS, Sousa VP, Oshima W, Ferro JA, Reinach FC. Concerted action of the high affinity calcium binding sites in skeletal muscle Troponin C. *J Biol Chem* 1995;270:9770-7.
10. Kobayashi T, Grabarek Z, Gergely J, Collins JH. Extensive interactions between troponins C and I. Zero-length cross-linking of troponin I and acetylated troponin C. *Biochemistry* 1995;34:10946-52.
11. Pearlstone JR, Smillie LB. Evidence for two-site binding of troponin I inhibitory peptides to the N and C domains of troponin C. *Biochemistry* 1995;34:6932-40.
12. Solaro J. Troponin C-troponin I interactions and molecular signaling in cardiac myofilaments. In: Sideman S, Beyar R, eds. *Molecular and subcellular cardiology: effects of structure and function*. New York: Plenum Press, 1995:109-15.
13. Adams JE, Schechtman KB, Landt Y, Ladenson JH, Jaffe AS. Comparable detection of acute myocardial infarction by creatine kinase MB isoenzyme and cardiac troponin I. *Clin Chem* 1994;40:1291-5.
14. Adams JE, Bodor GS, Davila-Roman VG, Delmez JA, Apple FS, Ladenson JH, Jaffe AA. Cardiac troponin I, a marker with high specificity for cardiac injury. *Circulation* 1993;88:101-6.
15. Larue C, Calzolari C, Bertinchant JP, Leclercq F, Grolleau R, Pau B. Cardiac-specific immunoenzymometric assay of troponin I in the early phase of acute myocardial infarction. *Clin Chem* 1993;39:972-9.
16. Jaffe A, Landt Y, Parvin CA, Abendschein DR, Getman AM, Ladenson JH. Comparative sensitivity of cardiac troponin I and lactate dehydrogenase isoenzymes for diagnosing acute myocardial infarction. *Clin Chem* 1996;42:1770-6.

17. Malr J, Larue C, Mair P, Balogh D, Calzolari C, Puschendorf B. Use of cardiac troponin I to diagnose perioperative myocardial infarction in coronary artery bypass grafting. *Clin Chem* 1994;40:2066-70.
18. Adams JE, Sicard GA, Allen BT, Bridwell KH, Lenke LG, Davila-Roman VG, et al. Diagnosis of perioperative myocardial infarction with measurements of cardiac Troponin I. *New Engl J Med* 1994;330:670-4.
19. Adams JE, Davila-Roman VG, Bessey PQ, Blake DP, Ladenson JH, Jaffe AS. Improved detection of cardiac contusion with cardiac Troponin I. *Am Heart J* 1996;131:308-12.
20. Etievent JP, Chocron S, Toubin G, Taberlet C, Alwan K, Clement F, et al. Use of cardiac troponin I as a marker of perioperative myocardial ischemia. *Ann Thorac Surg* 1995;59:1192-4.
21. Chocron S, Alwan K, Toubin G, Clement F, Kaiti D, Taberlet C, et al. Crystalloid cardioplegia rate of delivery and cardiac troponin I release. *Ann Thorac Surg* 1996;62:481-5.
22. Panteghini M, Bonora R, Pagani F, Buffoli F, Cuccia C. Rapid, highly sensitive immunoassay for determination of cardiac troponin I in patients with myocardial cell damage. *Clin Chem* 1997;43:1464-5.
23. Katrukha AG, Bersznikova AV, Esakova TV, Petterson K, Lovgren T, Severina ME, et al. Troponin I is released in bloodstream of patients with acute myocardial infarction not in free form but as complex. *Clin Chem* 1997;43:1379-85.
24. Pichon H, Chocron S, Alwan K, Toubin G, Kaiti D, Falcoz P, et al. Crystalloid versus cold blood cardioplegia and cardiac troponin I release. *Circulation* 1997;96:316-20.
25. Larue C, Defacque-Lacquement H, Calzolari C, Le Nguyen D, Pau B. New monoclonal antibodies as probes for human cardiac troponin I: epitopic analysis with synthetic peptides. *Mol Immunol* 1992;29:271-8.
26. IUPAC Analytical Chemistry Division. Nomenclature, symbols, units and usage in spectrochemicals analysis-II, data interpretation. *Anal Chem* 1976;48:2294-6.
27. Pardue H. The inseparable triad: analytical sensitivity, measurement uncertainty, and quantitative resolution. *Clin Chem* 1997;43:1831-37.
28. Larue C, Marquet PY, inventors. Stabilized composition of troponin for immunoassays and process for stabilizing troponin for immunoassays. US patent 5,583,200, 1996; French patent 93 02067. 1993.